Story of the Fish Screening Assay
in EPA's Endocrine Disruptor Screening Program
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Background

The story of the fish screening assay is a complex one, as it has proceeded through various stages of protocol development, prevalidation, and validation both in the U.S. and in the international arena with the Organization of Economic Cooperation and Development (OECD). The purpose of the fish screening assay is to detect estrogen- and androgen-active materials in fish. A secondary purpose of this assay involves its capacity to evaluate other hormonal and non-hormonal interactions with the reproductive systems of fish. The fish screening assay uses intact mature fish to examine abnormalities associated with survival, reproductive behavior, secondary sex characteristics, histopathology, and fecundity (i.e., number of spawns, number of eggs/spawn). As a screening assay it is not intended to confirm or quantify these effects or their mechanisms of action. The assay is being considered for inclusion in the Tier 1 screening battery to capture chemicals that potentially interact with the hormone systems of fish. Fish species occupy one end of the vertebrate spectrum, and the assay should be considered an integral component for inclusion in the Tier 1 screening battery.

Protocol Development

The Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) originally proposed a fish gonadal recrudescence protocol to be used as a fish screening assay. An EPA Office of Research and Development (ORD) laboratory undertook a feasibility study to determine whether this protocol would adequately detect estrogen- and androgen-active chemicals. The ORD laboratory selected the fathead minnow as the test species, as a large amount of historical data and experience were available regarding the use of this species in toxicity tests. The results of these feasibility studies indicated that the fish gonadal recrudescence protocol was not an effective test paradigm for a small continuous spawning species like the fathead minnow due to high variability in the gonadal recrudescence response.

Consequently, EPA sought to develop a new protocol to fulfill this need. There was some initial consideration for the evaluation of thyroid effects, but it was determined that it would be difficult to combine thyroid disruption and estrogen and/or androgen relevant disruption for fish in the same short-term assay. Furthermore, other assays in the Tier 1 screening battery (e.g., amphibian metamorphosis) are better able to detect thyroid effects. In developing a new fish screening protocol, EPA identified certain endpoints it believed to be indicative of estrogen and androgen effects. These endpoints included vitellogenin induction (indicative of an estrogen agonist/antagonist) and secondary sex characteristics or fish morphology (indicative of an androgen agonist/antagonist). In the case of the fathead minnow, secondary sex characteristics include color changes and the appearance of adipose fatty patches on the male during breeding season. Tubercles, which aid in mating and nest building, also form on the forehead of the male fathead minnow and, like the fatty patches, are under the influence of

androgen. Females exposed to androgen agonists often exhibit adipose tissue and tubercle development as well. In addition to these endpoints, fecundity measures were evaluated as indicators of hormonal disturbance, and gonadal histopathology was considered as a measurement capable of assisting in interpreting whether any observed fecundity changes are due to hormonal or non-endocrine systemic effects. Measuring circulating sex steroid content was also considered for detecting changes in synthesis or transport of hormones in the fathead minnow.

Protocol Evaluation

After identifying these endpoints, EPA developed a Detailed Review Paper (DRP) discussing fish screening protocols potentially capable of detecting estrogen and androgen effects. Four protocols were explicitly discussed in the DRP, and these protocols differed largely by the endpoints and life stages they examined. One evaluated the induction of vitellogenin in juvenile fishes; another considered using a more mature phase of the fish in a non-reproductive form; and two others examined the fish in a spawning stage (e.g., short term fish reproduction protocols). The EPA DRP was submitted to the OECD which adapted it as its own DRP for the fish screening assay. The OECD DRP included an expanded discussion of alternative protocols, including a sexual differentiation assay, or partial life cycle, to evaluate sexual differentiation from the embryo to juvenile life stages.

Certain of the protocols discussed in the DRPs were dismissed from further consideration due to inherent limitations. The juvenile fish protocol evaluating the induction of vitellogenin, for instance, did not allow for the detection of androgen-active compounds. In another example, certain endpoints used in the non-spawning protocol (e.g., gonadal histopathology) were not as informative due to a lack of responsiveness and sensitivity in the non-reproductive stage. In addition, the partial life cycle/sexual differentiation protocol proved to be too lengthy for a screening assay, lasting 60 days or longer, and would not necessarily be as efficient or effective as the fish reproduction protocol.

Following the development of the DRPs, EPA chose to investigate two versions of the short term fish reproduction protocol: the full version, with a 21-day exposure period, and an abbreviated version, with a 14-day exposure period. EPA prefers the 21-day fish reproduction protocol but wanted to explore whether an abbreviated version may be sufficiently effective. The Endocrine Disruptor Methods Validation Subcommittee (EDMVS) provided input regarding the preferred protocol for the fish screening assay. Some members were concerned that the 21-day fish reproduction protocol was too lengthy and complicated and that the fecundity measures could lead to false positives because the observed response could be due to non-endocrine, systemic mechanisms. Others fully supported the reproduction assay because it was more comprehensive, and its ability to detect estrogen and androgen effects has been demonstrated. EDMVS's general acceptance, although not a full endorsement, of the full version of the short term fish reproduction assay, led EPA to continue validating the 21-day version.

Prevalidation

An ORD laboratory (MED, Duluth, MN) undertook the task of determining whether the short term fish reproduction protocol could provide a truly robust test paradigm, and it initially used four types of compounds to test the protocol's ability to detect estrogen and androgen effects. These compounds were chosen by experts and included an estrogen agonist, aromatase inhibitor, androgen, and anti-androgen. The ORD laboratory demonstrated that the protocol was responsive for the mechanisms and endpoints as anticipated. The next step in the process involved demonstrating the transferability of the protocol to another laboratory. A Battelle laboratory, under contract to EPA, was able to replicate the results with these same four compounds.

At this point, the OECD became more directly involved in examining the fish screening assay. While they did consider the EPA-developed short term fish reproduction protocol, they also entertained a juvenile fish protocol and later, a non-spawning fish protocol (as described in the DRPs). The OECD, after two Fish Expert Consultations, identified three core endpoints to be initially considered. The OECD sought to evaluate these endpoints which included vitellogenin induction, gonadal histology, and secondary sex characteristics/morphology (e.g., gonado-somatic index). To this end, the U.S. agreed to initiate a vitellogenin survey to examine a variety of existing methods to quantify vitellogenin and to ascertain whether there was a need to standardize the methodology used to measure this endpoint. The U.S. also conducted a comparative evaluation of alternative versions of the fish screening assay, including the 21-day and 14-day versions of the short term fish reproduction protocol and the non-spawning version using the fathead minnow as the test species. Other countries evaluated the medaka and the zebrafish using their own preferred protocols.

In general, protocol development and prevalidation both within the U.S. and in the international arena has taken longer than expected. Shifting from the gonadal recrudescence to the development of a reproduction protocol was a lengthy exercise, and coordinating the DRP through OECD led to the consideration of alternative test species (e.g., the medaka and zebrafish).

The OECD undertook a Phase 1A validation trial to investigate the feasibility and practicality of conducting the fish assay in an international context. All three fish species were included in the trial, and four labs participated, each using two of the three species. Mature males and females were kept in separate tanks to prevent spawning. The results from the Phase 1A trial were presented to EDMVS, and these results established that the gonadal somatic index was not a reliable endpoint in these fish species and test conditions. Furthermore, examining gonadal histopathology was troublesome in non-spawning fishes because female fish exhibited pathology due to their non-spawning status. Likewise, morphological endpoints in non-spawning fishes were similarly considered inappropriate. Among the three species, the fathead minnow and medaka both have strong secondary sex characteristics in actively spawning

condition, but the zebrafish does not. Whether the zebrafish will be used in the final OECD protocol will depend upon its ability to respond sufficiently with just the vitellogenin and histopathology endpoints. The Phase 1A validation trial ultimately concluded that mature fish in the spawning stage were necessary for a fully effective assay.

This assortment of pre-validation studies and validation trials helped to optimize several aspects of the fish screening assay. The three fish species used in the various protocols have been employed extensively in past laboratory studies; therefore, optimization was not necessary to determine ideal testing conditions. However, the life stages and endpoints being examined still needed to be standardized and optimized. While the OECD agreed that the gonado-somatic index was not a useful endpoint, the methods used to measure vitellogenin induction, gonadal histopathology, and morphological changes required additional study and discussion. For instance, a choice had to be made among a variety of commercial kits available to measure vitellogenin, and the special studies helped to determine how to uniformly quantify the appearance of adipose tissue and tubercles on fathead minnows and anal fin development on the medaka.

Validation

Following Phase 1A, the next international validation trial, Phase 1B, tested a modified version of the short term reproduction assay. In this modified assay, mature male and female fish in spawning condition were to be held in groups in the same tank and fecundity was measured qualitatively (yes/no). A test matrix was designed in which 14 laboratories carried out the protocol using one of three fish species and two of three pre-chosen compounds. While exposures have been completed, the final report is still under review and subject to revision. In general, the Phase 1B trial demonstrated that a 21-day assay with the three species is able to detect a weak estrogen and an aromatase inhibitor through measurement of vitellogenin, and an anti-androgen through histological changes in the gonads. The results also suggested that more appropriate spawning conditions are needed, especially for the fathead minnow. It was also suggested that quantitative data on fecundity may be useful and that this endpoint should be further explored. EPA agreed to proceed with a follow-on study to the Phase 1B to address these suggestions. The follow-on study will include a repeat with the anti-androgen used in Phase 1B, a steroidogenesis inhibitor, and a toxic negative. The protocol will be modified to expose fish in biologically appropriate spawning groups (as described in EPA's short term reproduction protocol) and to collect quantitative fecundity data.

In addition to the OECD Phase 1B and follow-on work, EPA must also consider the results of a U.S. study undertaken contemporaneously with the Phase 1B trial, which consisted of a multi-chemical study using the full 21-day version of the short term fish reproduction assay. Six compounds were chosen to determine the responsiveness of the assay. Because optimization, prevalidation, and validation have overlapped among the protocols and domestic/international efforts, EPA will solicit the Endocrine Disruptor Methods Validation Advisory Committee's (EDMVAC) opinion on the next step regarding the validation of the fish screening assay.